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The cell adhesion molecule CD44 is encoded by a complex gene and undergoes extensive alternative splicing. Differential regulation of CD44 splicing has been implicated in human tumorigenesis, and differences in variant isoform expression in normal versus cancerous breast tissue suggest a role for CD44 in the progression of breast cancer. Our preliminary analysis of CD44 expression in human breast samples by RT-PCR and Southern blot hybridization revealed no significant correlation between breast tumor type and CD44 variants. The retention of CD44 intron 9 was however demonstrated in 50% of breast tumors examined, supporting the hypothesis that dysregulation of CD44 splicing accompanies tumorigenesis. Novel CD44 isoforms containing single variant exons were identified in a related study of primary and metastatic tumors of the central nervous system. Analysis of similar variant isoforms in breast tumors may offer some insight into breast cancer metastasis. Examination of CD44 splicing in the human breast cancer cell lines BT-20, MDA-MB-435s and ZR-75-1 resulted in the cloning of a novel CD44 variant isoform generated by multiple splicing events. Differential usage of splicing signals associated with CD44 variant exons v2 and v3 was demonstrated in these cell lines and should allow for further delineation of exon sequences that regulate CD44 splicing in breast cancer.

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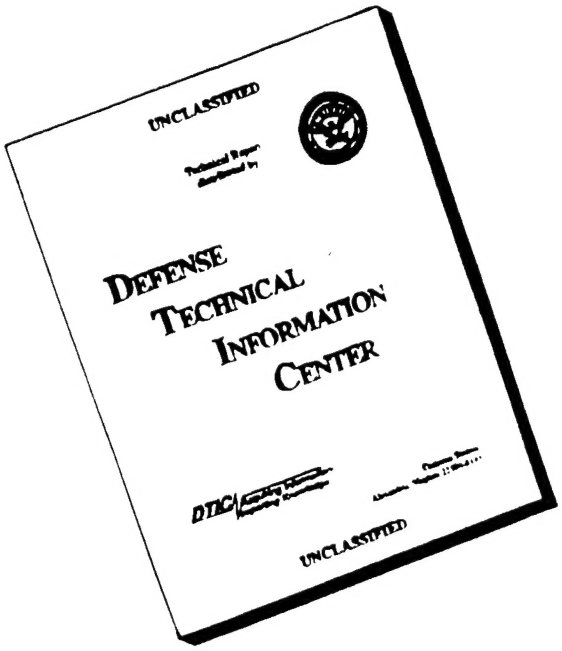

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Nicole M. Resnick 8-26-96

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INTRODUCTION

The cell adhesion molecule CD44 is a cell surface transmembrane glycoprotein which exhibits a wide range of biological functions. CD44 is involved in the processes of lymphocyte homing and activation, inflammation, and it serves as a receptor for hyaluronan, chondroitin-4-sulfate, and a number of proteoglycans that possess chondroitin sulfate side chains (1-6). Because CD44 is able to bind ligands of the extracellular matrix, it is hypothesized that CD44 may play a role in cell-matrix and cell-cell adhesion. Although originally identified in lymphocytes, CD44 expression has since been documented in a wide variety of cell types and tissues including breast, lung, colon, brain and prostate (7).

The CD44 gene is approximately 60 kilobases long and encodes a complex transcription unit comprised of at least twenty exons. The heterogeneity of the CD44 protein was originally believed to be due to extensive modification of the protein including O- and N-linked glycosylation, as well as chondroitin sulfation. However, genomic and cDNA cloning and sequencing of the CD44 gene revealed that the molecule's diversity is also attributed to the RNA processing mechanism of alternative splicing (8-11). Twelve of the twenty exons are subject to alternative splicing, and ten of these exons (v1-v10) are spliced at a unique extracellular site to generate numerous CD44 isoforms. The two most prevalent isoforms of CD44, CD44s and CD44R, were first identified on cells of hematopoietic and epithelial origin respectively. CD44R was the first alternatively spliced form of CD44 identified - it contains an additional 132 amino acids, encoded by variant exons v8-v10, spliced into the ten framework exons of CD44s (see Figure 1, Appendix). CD44 isoforms containing any other combination of variant exons are collectively designated CD44 variants (CD44v).

Alternative splicing of the CD44 gene to produce variant isoforms has been implicated in the processes of tumorigenesis and metastasis. Some of the earliest immunohistochemical and RT-PCR (reverse transcription-polymerase chain reaction) analyses have demonstrated that while the CD44R transcript is weakly expressed in some normal epithelium, expression is greatly increased in human carcinomas (12-13). A compelling study conducted in rat pancreatic carcinoma cell lines demonstrated that transfection of a particular CD44 isoform comprised of several variant exons served to induce metastasis in cells normally unable to metastasize (14). Human homologues of the rat metastatic clone have been identified in tumor specimens, and homologues of variant CD44 transcripts expressed in a rat mammary carcinoma metastatic cell line have been detected in human breast cancer

cell lines (15-16). Thus, differential regulation of the alternative splicing of CD44 may play a role in the pathogenesis of breast cancer.

More recent studies of CD44 in human tumors suggest that CD44 expression may serve more accurately as a marker of cellular differentiation, rather than tumor progression. This shift in hypothesis is supported by data from a large immunohistochemical analysis of 327 breast carcinomas using monoclonal antibodies specific for CD44 variant exons (17). Patient survival did not correlate with the expression of particular variant exons; instead, CD44s, variant exon v6 and variant exon v9 immunoreactivity served as markers for cellular differentiation. A similar study of renal cell carcinomas demonstrated that variant exon expression strongly correlated with tumor grade. Terpe and colleagues (18) reported a significant increase in CD44s and CD44v expression in the course of tumor differentiation in clear cell and cromophobe cell carcinomas. In another immunohistochemical study of primary lung tumors, total CD44 levels were consistently high among the better differentiated, less aggressive non small cell lung carcinomas, compared to low levels of CD44 expression in undifferentiated small cell carcinomas (19). The investigators of this study concluded that in lung cancers, CD44 is associated with specific differentiation phenotypes. In a lung tumor analysis conducted by our laboratory, a similar result was demonstrated (Resnick *et al.*, manuscript in preparation).

The purpose of this research is to investigate the role of CD44 in human breast cancer. We initially proposed to examine the expression of the alternatively spliced CD44 isoforms in human breast tissue to test the assumption that differences in alternative splicing of the CD44 transcript exist between normal breast and tumor breast tissue. Primary and metastatic breast specimens were processed for RNA and then examined for CD44 expression by RT-PCR and Southern blot methodology. Our preliminary results comparing matched normal and tumor samples from resected breast tissue indicated that tumor tissue exhibits differences in CD44 expression. These differences are both quantitative and qualitative, in that tumors express an overall greater amount of CD44 message, and the transcripts are larger in size and contain more variant exons than normal breast tissue.

Another specific aim of our proposal is to investigate the molecular signals and mechanisms responsible for regulating the alternative splicing of CD44 in human breast cell lines. We would like to determine what is responsible for the differential regulation of CD44 alternative splicing detectable by RT-PCR and immunohistochemistry between normal and

breast tumor specimens. Upon elucidating the molecular signals involved, we can begin to understand how their regulation plays a role in the invasive potential and metastatic proclivity of human breast cancer cells.

Specific Aim 1: To examine the expression of alternatively spliced CD44 isoforms in human breast cancer

A) Analysis of CD44 variant isoform expression in human tumor tissue by RT-PCR and Southern blot methodology

By the completion of the first year of funding, a total of 99 human breast tissue specimens, including normal and cancer tissue, were analyzed by RT-PCR and Southern blot hybridization. Our results suggested there was not a significant correlation between CD44 variant isoform expression and human breast cancer. Although in 47% (16/34) of the matched sets of tumor and normal breast tissue the tumors expressed overall higher levels of CD44 variant isoforms, containing multiple variant exons, than normal breast specimens, no association between breast tumor type and CD44v expression could be made. Approximately 40% of normal breast tissue was found to express CD44 isoforms larger in size than CD44s, a finding that is consistent with more recent human breast tissue studies since our initial proposal was formulated (20-21). Furthermore, the number of breast specimens we obtained from our institution decreased significantly, precluding our ability to assess the number of samples originally deemed necessary to conduct a complete prospective study as outlined in the initial research proposal.

For these reasons, we decided to focus our efforts on studying CD44 variant isoform expression in human breast cell lines. The BT-20, MDA-MB-435s, and ZR-75-1 cell lines (all obtained from the ATCC, Rockville, MD) have already been characterized in our laboratory with respect to CD44 isoform expression. By studying the relationship between CD44 splicing and the invasive properties of these cell lines, we believe we may better understand the role played by CD44 in human breast cancer metastasis.

Before redirecting our focus of CD44 expression in human breast cancer, we investigated one other relatively new finding related to CD44 splicing in human tumors. Dysregulation of CD44 splicing in certain human cancers has now been shown to result in the retention of introns, which are normally spliced out of pre-mRNA molecules prior to RNA expression (22-23). The absence of intron retention in matched normal tissues has suggested to

investigators that aberrant intron expression may serve as a marker in particular cancers. Because the specific expression of CD44 intron 9 in human bladder cancers was brought to our attention by our collaborator Dr. David Tarin of Oxford University, we designed a probe to CD44 intron 9 and hybridized a subset of our breast cancer and normal breast tissue samples. Out of six breast tumor samples examined, 50% showed expression of intron 9 while 0/6 matched normal breast tissues exhibited positive hybridization (see Figure 2, Appendix). Because this result substantiates the findings of others, we are interested in testing its significance in human breast cell lines.

B) Novel CD44 variant isoform cloned from the BT-20 breast cancer cell line

A novel CD44 variant isoform cloned by us just prior to submission of last year's annual report was verified by sequencing. As reported one year ago, a CD44-specific primer designed to amplify variant exon v3-containing isoforms from breast cancer cell lines was used to clone a PCR product from BT-20 cells. Sequencing of the 340 basepair fragment by standard procedures (Sequenase, USB) confirmed the exon composition. In this BT-20 cell-specific variant isoform, CD44 standard exon 5 is spliced directly to variant exon v3, which is then spliced directly to variant exon v8. Variant exons v9 and v10 are also included (see Figure 3, Appendix). Due to this unusual splicing process, variant exon v2 is deleted, as well variant exons v4 - v7. Another investigator has reported the identification of this CD44 isoforms in a metastatic breast tumor sample (24). Although the biologic role(s) of this isoform has yet to be determined, its differential synthesis in human breast cancer cell lines strongly suggests that the CD44 splicing machinery is subject to tissue-specific controls. The isolation and sequencing of this novel clone provides us with an additional tool in the laboratory to study the role of particular CD44 variant exons in tumorigenesis.

C) CD44 Variant Exon Expression in Human Central Nervous System Tumors

Because this past year we entered into a collaboration with investigators working on tumors of the central nervous system, we decided to apply our expertise in RT-PCR/Southern blot methodology to the analysis of CD44 variant exon expression in tumors of the central nervous system (CNS). Our rationale for pursuing this line of research, despite our primary focus on human breast cancer, is that identification of particular CD44 expression

patterns in primary versus metastatic tumors of the brain and spine might shed some light on the role of CD44 in metastasis of breast tumors. Using the same protocol as that described for the study of human breast tissues, a total of 56 CNS samples were analyzed. Briefly, tissue from primary or metastatic tumors was homogenized for extraction of RNA. A set of human CNS cell lines, including normal, primary tumor and metastatic tumor cell lines established from tissue specimens, was also processed for RNA by standard procedures. RNA was then converted into double-stranded cDNA by reverse transcription according to previously described methods. Amplification of the cDNA was carried out using CD44 gene-specific primers which anneal to framework exons outside of the ten variant exons (see Figure 1, Appendix). All PCR products were then resolved by agarose gel electrophoresis, Southern blotted onto nylon membranes, and sequentially hybridized with a probe corresponding to each individual CD44 variant exon v3 through v10 to more accurately determine variant exon expression. Hybridization and detection was carried out using the non-radioactive enhanced chemiluminescence system (ECL, Amersham).

Analysis of CD44 variant expression in tissues of the CNS revealed several interesting findings: (1) although tumors metastatic to the brain exhibit high levels of CD44 variant isoform expression containing multiple variant exons, tumors metastatic to the spine, in contrast, exhibit very low-level expression of CD44 variant exons (see Figure 4, Appendix); (2) tumors metastatic to the spine, as well as some primary brain tumors and cell lines established from normal brain and primary brain tumors exhibit low level expression of CD44v containing *single* variant exons. These single variant exon-containing isoforms have been documented in several other human cancers (17-18, 25-26). Their recent identification is sparking increased interest in the roles these small CD44 isoforms may play in human tumorigenesis. We are the first to demonstrate these isoforms in human tumors of the CNS, and based on these results, we intend to examine the expression of similar amplification products in human breast tissue. Furthermore, upon cloning or synthesizing these novel CD44 products, we can introduce them into breast cells in culture and study any subsequent changes in invasion, tumorigenicity or interaction with ligands of the ECM.

Specific Aim 2: To investigate the molecular mechanisms regulating the alternative splicing of CD44 in human breast cell lines

A) Identification of CD44 splicing signals

Our initial strategy for identifying the cis- and trans-acting splice signals which mediate alternative splicing of the CD44 gene involved introducing CD44 minigene constructs into breast cell lines. The transfected cells were then intended to be fused to form heterokaryons, and CD44 splicing activity in the heterokaryons would be compared to the unfused parental cells. The minigene constructs contain an alkaline phosphatase gene which serves as a reporter to measure splicing activity in cells. As reported one year ago, this part of the study was initiated with the transient transfection of the minigene constructs into breast cell lines BT-20, MDA-MB-435s and ZR-75-1. Efforts to demonstrate expression of the alkaline phosphatase reporter gene were unsuccessful although the positive control transfections using a *lacZ* gene in these cells showed β–galactosidase activity by a colorimetric assay.

Our laboratory has since obtained new minigene splicing constructs cloned into the pCEP4 vector (Invitrogen) which contains the CMV (cytomegalovirus) immediate early promoter, as opposed to the pREP9 vector which uses the RSV (Rous sarcoma virus) promoter to drive gene expression. Despite repeated attempts to demonstrate expression of alkaline phosphatase in breast cell lines transiently transfected with the new splicing constructs, the experiments have not yet worked. The conditions for transfections, as well as detection of alkaline phosphatase activity have been modified in a variety of ways in attempts to overcome this technical problem. Since alkaline phosphatase activity using these constructs has yet to be demonstrated in any human breast cell lines, we conclude that an alternative reporter gene should be used to study the splicing signals responsible for the alternative processing of CD44 pre-mRNA in human breast cells.

In order to continue pursuing the identification of CD44 splice signals in human breast cell lines, we devised an alternative strategy which incorporates our expertise with RT-PCR in these cell lines. To first demonstrate that different human breast cancer cell lines are subject to specific splicing controls, we focused our attention on the splicing of the first two variant exons v2 and v3. CD44 variant exon-specific primers were designed which specifically anneal to each of these exons. When either of these sense primers was used in combination with an antisense primer specific to variant exon v10 in a PCR reaction, amplification

products from the three human breast cell lines revealed differences indicative of cell line-specific splicing controls (see Figure 5, Appendix). We conclude from these studies that CD44 splicing events are indeed under the control of specific signals which differ in the various cell lines tested. Precedence for this finding was established by our initial demonstration of differences in the basic CD44 isoform profiles in each of these cell lines using the standard CD44 primers.

We intend to further investigate both the cis- and trans-acting control signals which account for the differences in CD44 variant exon splicing in cell culture. By cloning the sequences surrounding the v2 and v3 splice junctions we can better delineate the exonic regions responsible for splicing control. Exon-splicing enhancers (ESEs) have recently gained recognition as one of the factors that may affect splice site choice in the differential splicing of vertebrate pre-mRNAs (27-28). These exonic sequences are usually purine-rich, have a general consensus of GARGARGAR (R=purine) and have been observed to serve as binding sites for SR proteins (29). Genomic sequences within CD44 exons that contain the ESE consensus sequence have been identified by our colleagues at the Terry Fox Cancer Center in Vancouver, BC. Through computer sequence analyses of conserved ESEs and the CD44 gene, we hope to confirm these findings and then perturb these sequences by site-directed mutagenesis to test their role in CD44 alternative splicing.

CONCLUSIONS

As indicated in last year's Annual Report, a redirection of our strategy to investigate the regulation of CD44 expression in human breast cancer is warranted. Our realization of the limitations of studying CD44 variant isoforms in human breast tissue specimens by RT-PCR and Southern hybridization is strongly supported by the recent findings of our colleagues in the field of CD44. Advances in the technology used to analyze CD44 variant expression have allowed researchers to attain higher levels of specificity. The widespread use of highly specific monoclonal antibodies in immunohistochemical analyses of CD44 variant expression, coupled with more sophisticated PCR techniques and variant-exon specific hybridization of PCR products strongly demonstrates the complexity of CD44 gene regulation in human breast cancer. Most significant is the growing consensus that CD44 variant exon expression does not necessarily correlate with outcome or prognosis in human cancer. The newly emerging hypothesis that CD44 expression is associated with cellular differentiation will most likely change the way investigators tackle the problem of understanding CD44's role in tumorigenesis.

Analysis of CD44 gene expression in human breast cancer cell lines should continue to provide us with a useful model system. We have already demonstrated the value of these cell lines in identifying novel CD44 variant isoforms. The manipulation of variant isoform clones will allow us to more precisely target regions of the CD44 gene, both intronic and exonic, that control differential splicing. In this past year we have come to appreciate the potential role of exon-splicing enhancers, as well as the phenomenon of intron retention in human tumor samples. By studying these gene regulatory features in a more controlled in vitro cell culture model, we may gain a better understanding of their role in human breast cancer. Ongoing progress in our laboratory in the areas of ribozyme-mediated inhibition of gene expression and utilization of CD44 splicing control signals in a chimeric enzyme/prodrug therapy (CEPT) to target breast and brain metastases will further ensure that this investigation of CD44 gene regulation in breast cancer will achieve worthwhile results.

REFERENCES

1. Goldstein, L.A., Zhou, D.F.J., Picker, L.J., Minty, C.N., Bargatze, R.F., Ding, J.F., Butcher, E.C. (1989) *Cell* 56,1063-1072.

2. Jalkanen, S., Bargatze, R.F., de los toyos, J., Butcher, E.C. (1987) *Journal of Cell Biology* 105,983-990.

3. Miyake, K., Medina, K.L., Hayashi, S., Ono, S., Hamaoka, T., Kincade, P.W. (1990) *Journal of Experimental Medicine* 171, 477-488.

4. Aruffo, A., Stamenkovic, I., Melnick, M., Underhill, C.B., Seed B. (1990) *Cell* 61, 1303-1313.

5. Toyama-Sorimachi, N., Sorimachi, H., Tobita, Y., Kitamura, F., Yagita, H., Miyasaka, M. (1995) *Journal of Biological Chemistry* 270,7437-7444.

6. Naujokas, M.F., Morin, M., Anderson, M.S., Peterson, M. , Miller, J. (1993) *Cell* 74, 257-268.

7. Haynes, B.F., Telen, J.J., Hale, L. P., Denning, S.M. (1989) *Immunology Today* 10, 423-428.

8. Cooper, D.L, Dougherty, G., Harn, H.-J., Jackson, S., Baptist, E.W., Byers, J., Datta, A., Phillips, G., Isola, N. (1992) *Biochemistry and Biophysical Research Communications* 182, 569-578.

9. Dougherty, G.J., Lansdorp, P,.M., Cooper, D.L., Humphries, R.K. (1991) *Journal of Experimental Medicine* 174, 1-5.

10. Jackson, D.G., Buckley, J., Bell, J.I. (1992) *Journal of Biological Chemistry* 267, 4732-4739.

11. Screaton, G.R., Bell, M.V., Jackson, D.G., Cornell, F.R., Gunthert, U., Bell, J.I. (1992) *Proceedings of the National Academy of Science USA* 89, 12160-12164.

12. Tanabe, K.K., Ellis, L.M., Saya, H. (1993) *Lancet* 341, 725-726.

13. Matsumura, Y. and Tarin, D. (1992) *Lancet* 340, 1053-1058.

14. Gunthert U., Hofmann, M., Rudy, W., Reber, S., Zoller, M., Tolg, C., Ponta, H., Herrlich, P. (1991) *Cell* 65, 13-24.

15. Hofmann, M., Rudy, W., Zoller, M., Tolg, C., Ponta, H., Herrlich, P., Gunthert, U. (1991) *Cancer Research* 51, 5295-5297.

16. Heider, K.-H., Hofmann, M., Hors, E., van den Berg, F., Ponta, H., Herrlich, P., Pals, S.T. (1993) *Journal of Cell Biology* 120, 227-233.

17. Friedrichs, K., Franke, F., Lisboa, B.-W., Kugler, G., Gille, I., Terpe, H.-J., Holzel, F., Maass, H., Gunthert, U. (1995) *Cancer Research* 55, 5424-5433.

18. Terpe, H.-J., Storkel, S., Zimmer, U., Anquez, V., Fischer, C., Pantel, K., Gunthert, U. (1996) *American Journal of Pathology* 148, 453-463.

19. Penno, M.B., August, J.T., Baylin, S.B., Mabry, M., Linnoila, R. I., Lee, V.S., Croteau, D., Yang, X.L., Rosada, C. (1994) *Cancer Research* 54, 1381-1387.

20. Mackay, C.R., Terpe, H.-J., Stauder, R., Marston, W.L., Stark, H., Gunthert, U. (1994) Journal of Cell Biology 124, 71-82.

21.Dall, P., Heider, K.-H., Sinn, H.-P., Skroch-Angel, P., Adolf, G., Kaufmann, M., Herrlich, P., Ponta, H. (1995) *International Journal of Cancer* 60, 471-477.

22. Matsumura, Y., Hanbury, D., Smith, J.C., Tarin, D. (1994) *British Medical Journal* 308, 619-624.

23. Matsumura, Y., Sugiyama, M., Matsumura, S., Hayle, A.J., Robinson, P., Smith, J.C., Tarin, D. (1996) submitted.

24. Iida, N. & Bourguignon, L.Y.W. (1995) *Journal of Cellular Physiology* 162, 127-133.

25. Gunthert, U., Stauder, R., Mayer, B., Terp, H.-J., Finke, L., Friedrichs, K. (1995) *Cancer Surveys* 24, 19-42.

26. Rall, C.J.N., Rustgi, A.K. (1995) *Cancer Research* 55, 1831-1835.

27. Humphrey, M.B., Bryan, J., Cooper, T.A., Berget, S.M. (1995) *Molecular and Cellular Biology* 15, 3979-3988.

28. Tanaka, K., Watakabe, A., Shimura, Y. (1994) *Molecular and Cellular Biology* 14, 1347-1354.

29. Ramchatesingh, J., Zahler, A.M., Neugebauer, K.M., Roth, M.B., Cooper, T.A. (1995) *Molecular and Cellular Biology* 15, 4898-4907.

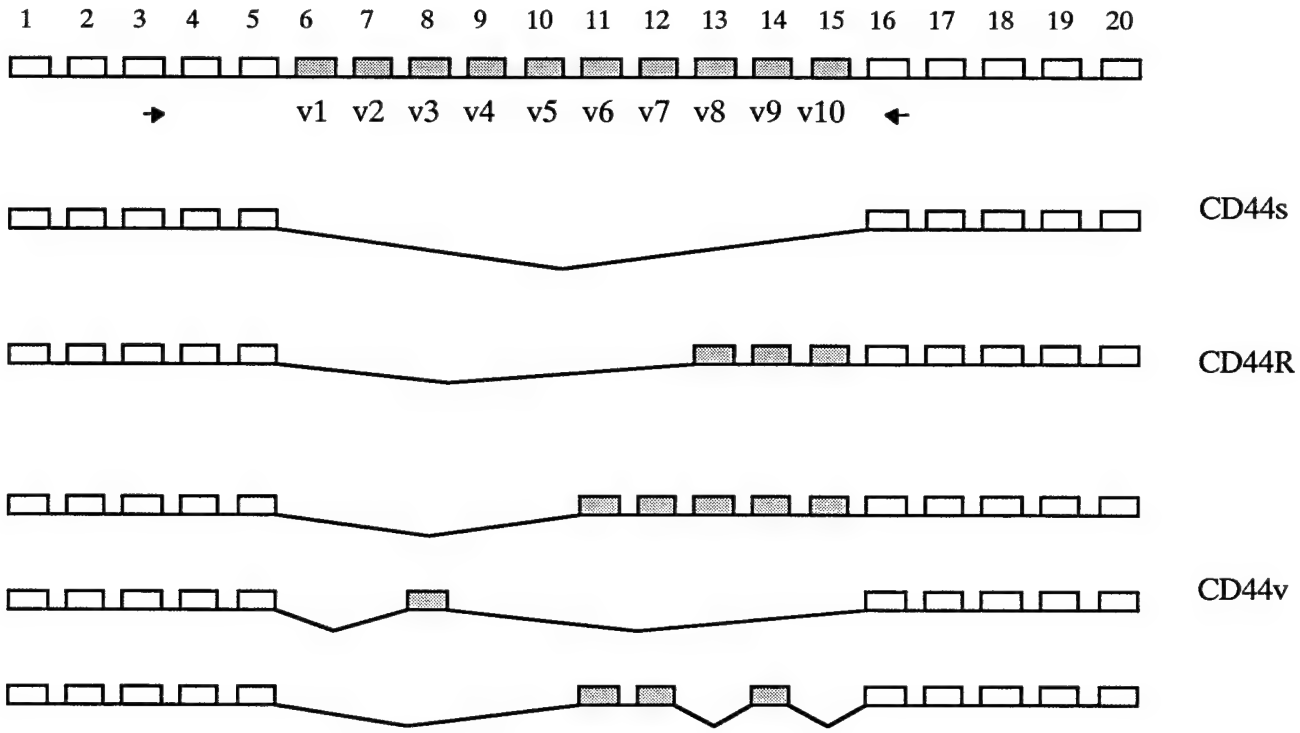

Figure 1: The Complex Transcription Unit of the CD44 Gene

The twenty exons comprising the CD44 gene are represented as boxes and are numbered. Numbers along the top denote the conventional exon numbering (1-20) and numbers along the bottom of the ten shaded boxes denote the variant exons (v1-v10) which undergo alternative splicing within the extracellular domain of the molecule. The positions where the two standard PCR primers used for RT-PCR anneal to the CD44 cDNAs are indicated by arrowheads. The two most prevalent CD44 isoforms generated by alternative splicing, CD44s and CD44R, are depicted, as well as several examples of CD44 variant isoforms (CD44v).

INTRON 9 RETENTION IN BREAST TISSUE

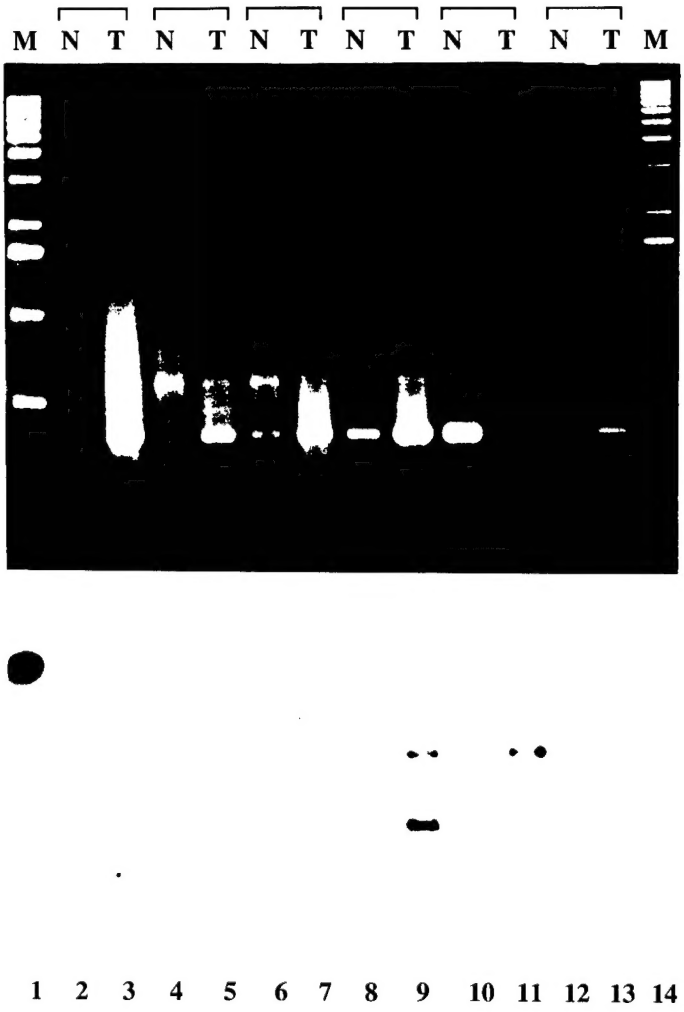

Figure 2: Intron 9 Retention in Breast Tissue

The top panel shows an ethidium bromide-stained agarose gel of CD44 PCR products amplified from six sets of matched human breast tissues. N denotes normal breast, T denotes tumor, and brackets indicated matched sets run side-by-side. M denotes molecular weight markers, and each lane is numbered along the bottom of the figure (1-14). The bottom panel is an autoradiograph of the agarose gel following Southern blotting and hybridization with a probe for CD44 intron 9. Positive hybridization signals are apparent in breast tumors in lanes 9 and 11. A third breast tumor (lane 3) also exhibited weak hybridization to the probe that is undetectable in this photograph.

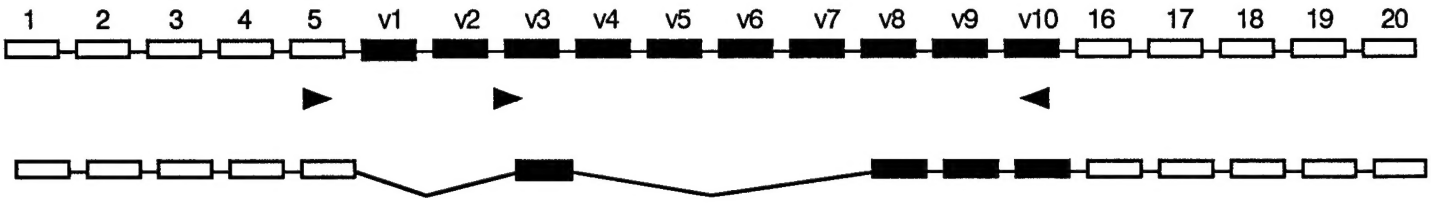

Figure 3: BT-20 Breast Cell Line Novel CD44 Variant Isoform

A schematic representation of the novel CD44 variant isoform cloned from the human breast cancer cell line BT-20 is shown. The twenty exons of the complete CD44 gene are depicted as boxes and numbered. The PCR primers used to amplify this clone are denoted by arrowheads below exons 5, v3 and v10 (the sense primer anneals to the 3' end of exon 5 juxtaposed directly to the 5' end of variant exon v3). The splicing events necessary to generate this isoform are indicated below the gene structure. A 340 basepair product was subsequently recovered from the PCR reaction and cloned. Sequencing revealed that it contains CD44 exons 5-v3-v8-v9-v10.

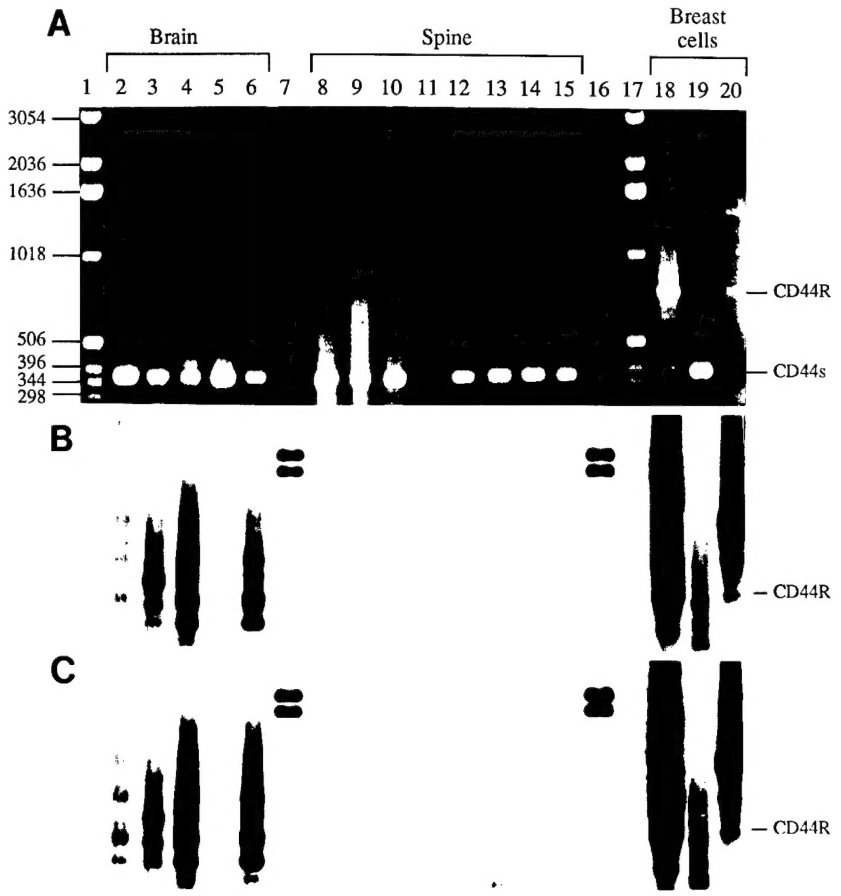

Figure 4: CD44 Expression in Human Tumors Metastatic to the Brain and the Spine

Panel A is an ethidium bromide-stained agarose gel of CD44 RT-PCR products amplified from a set of tumors metastatic to the brain (lanes 2-6), and tumors metastatic to the spine (lanes 8-15). Lanes 18-20 contain RT-PCR products from breast cell lines BT-20, MDA-MB-435s and ZR-75-1 respectively, and serve as positive controls for hybridization. Markers in lanes 1 and 17 are labeled in basepairs, and ECL-labelled markers run in lanes 7 and 16 are detectable in panels B and C following hybridization. CD44s and CD44R isoforms are denoted on the right-hand side.

Panel B is an autoradiograph of the gel following Southern blotting and hybridization to CD44 variant exon probe v4. Panel C shows subsequent hybridization to CD44 variant exon probe v6.

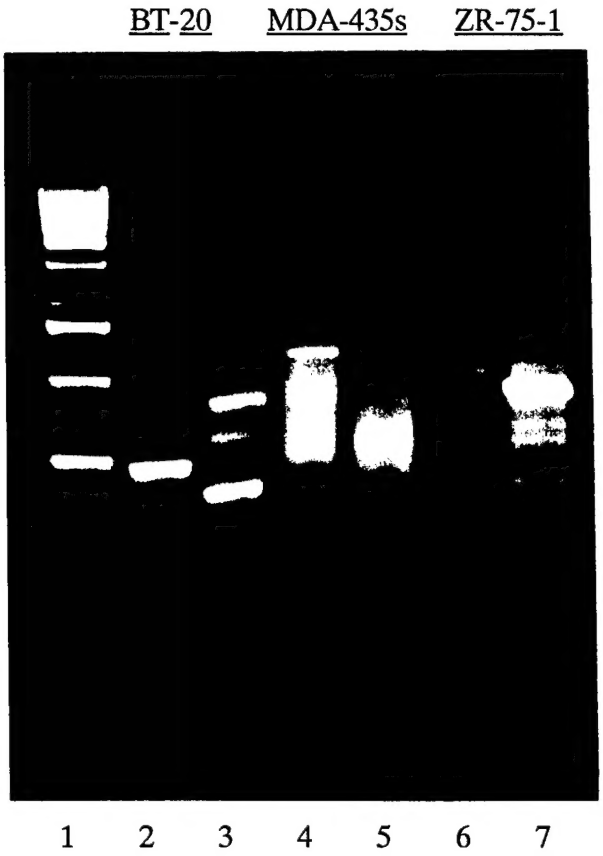

Figure 5: Human breast cancer cell lines exhibit differential splicing of CD44 variant exons v2 and v3

This ethidium bromide-stained agarose gel shows PCR products amplified from the three breast cancer cell lines BT-20 (lanes 2, 3), MDA-MB-435s (lanes 4, 5) and ZR-75-1 (lanes 6, 7) using either a v2 sense primer (lanes 2, 4, 6) or a v3 sense primer (lanes 3, 5, 7) in combination with an antisense primer to v10. Lane 1 contains molecular weight markers. The PCR product profiles differs for each of the cell lines, indicative of cell line-specific CD44 splicing control signals.